HILGARDIA

A JOURNAL OF AGRICULTURAL SCIENCE

PUBLISHED BY THE

CALIFORNIA AGRICULTURAL EXPERIMENT STATION

VOL. 6 JANUARY, 1932 No. 12

METHODS FOR THE ISOLATION OF BRUCELLA ABORTUS[1]

B. S. HENRY,[2] J. TRAUM,[3] AND C. M. HARING[4]

CONTENTS

[1] This study was in part supported by a grant from the United States Bureau of Animal Industry.

[2] Associate in Veterinary Science; resigned.

[3] Professor of Veterinary Science and Veterinarian in the Experiment Station.

[4] Professor of Veterinary Science and Veterinarian in the Experiment Station.

INTRODUCTION

Owing to the greatly increased interest in *Brucella abortus* since it has been definitely established as a human pathogen and since its economic importance has been more widely recognized, the attempted isolation of the organism from suspected materials has become a routine practice in a large number of laboratories not previously interested in the *Brucella* group.

The procedures given below, together with some experimental data, are based on several years' work of this and other laboratories interested in bovine-abortion control. For the most part the methods are those in general use or modifications of such. These methods have uniformly given satisfactory results in research and also in routine diagnosis such as certified-milk inspection and control work in other dairy herds. Because of these results, which have been obtained from the inoculation of several thousand guinea pigs, it was thought that a detailed description of the technique might be of value, in whole or in part, to other workers.

In addition to outlining the methods used, an attempt has been made to compare and evaluate the various procedures.

DIRECT CULTURE METHOD

Although the isolation of *Brucella abortus* by guinea-pig inoculation, especially from milk, has been the most widely accepted method for many years, from time to time the use of culture media for the direct isolation of the organism from suspected material has been advocated. The most recent work on this method is that of Huddleson, Hasley, and Torrey.[13] For the isolation of *Br. abortus* from milk, these workers use liver-infusion agar to which is added a saturated aqueous solution of gentian violet in the proportion of 1 part to 10,000 parts of culture medium. The medium is allowed to harden in petri dishes, and 0.1 cc of cream is spread over the surface of the plate. The dye inhibits the growth of most of the other bacteria in the milk, but allows *Br. abortus* to develop. The advantages of this method of isolation are numerous. The saving of time in reaching a final diagnosis, the low cost of plates as compared with the cost of guinea pigs, and the cost of their care and of the equipment necessary for maintaining them over a long period of time, as well as the time consumed in post-

mortem examinations, are all arguments in favor of the direct isolation by means of culture media. On the other hand, there has been a general feeling on the part of many laboratory workers that guinea-pig inoculation is the more delicate test for the detection of *Br. abortus,* especially in material containing other organisms.

Because of the lack of sufficient definite data on the relative merits of the two methods, it was decided to make cultures of milk from a large number of samples which were being used for guinea-pig inoculations.

The medium selected for this work was 'chocolate' or cooked-blood agar. This preparation, probably first described by Fleming[9] for the isolation of influenza bacilli, has been used in this laboratory since 1919, and has given excellent results in the primary isolation of *Brucella abortus* and many other organisms. Comparisons have been made from time to time between the cooked-blood agar and other media, particularly liver-infusion agar, but in all cases the cooked-blood agar has yielded the most uniform results.

Preparation of Culture Medium.—The cooked-blood agar is relatively simple to prepare, and it has a very definite advantage in that the constituents may be stored for a considerable length of time and fresh medium made in small quantities in a very short time. The variation between lots of this medium is relatively slight compared with infusion media. The method of preparation follows.

Two per cent of agar is added to ordinary nutrient broth, adjusted to a pH of 7.4, and sterilized. When the agar has cooled to below 70° C, 8 per cent by volume of sterile defibrinated horse blood is added. This mixture is placed in a water bath at 70° C for 10 minutes, after which it is transferred to a sterile covered funnel, to the end of which a rubber tube and pinch-cock has been attached. The medium is immediately distributed to sterile tubes and petri dishes.

Although the final pH will usually be about 6.8, a check may easily be made just previous to tubing the medium, by centrifuging a tube of the medium in a trunnion containing warm water, and testing the clear supernatant agar with an indicator.

For the isolation of *Brucella abortus* from material such as milk, which contains other organisms, 0.1 cc of a saturated alcoholic solution of gentian violet is added to every liter of medium, making a final dilution of the dye approximately 1 to 208,000.

Comparison of Culture and Guinea-Pig Injection.—The milk used for the comparative tests was taken from 22 cows, most of which

were positive to the agglutination test for *Brucella abortus*. The milk from each quarter of the udder was treated as a separate sample, and the results below represent from one to four sets of samples from each cow. The sample was centrifuged as described in the paragraph on preparation of material for injection, and the cream and sediment thoroughly mixed. The tubes containing the inoculum were kept iced except for a few minutes just previous to planting or injecting. At this time the material to be used was placed in a water bath at or slightly below 38° C, so that a representative sample could be obtained.

One-tenth cc of the material was placed on the surface of a gentian-violet cooked-blood-agar plate and spread over the surface by means of a wire dally. The plates were incubated in air overnight, then placed in 10 per cent carbon dioxide and incubated for four more days. At this time the *Brucella abortus* colonies were counted, and a typical colony from each positive plate transplanted, later being tested with known positive and negative *Br. abortus* antiserum. Suspicious colonies from plates having no typical *Br. abortus* growth were transplanted, and if the organisms resembled *Br. abortus* when stained, were also tested by agglutination.

The guinea pigs were inoculated intraperitoneally with 2 cc of the material, and slaughtered at the end of 6 weeks. The procedure outlined below for the post-mortem examination and isolation of *Brucella abortus* was followed.

A total of 247 parallel tests were made with plates and guinea-pig inoculation. Of these, 100 proved positive for *Brucella abortus* by one or both methods.

One hundred guinea pigs proved to be infected, and *Brucella abortus* was recovered from the spleen in each case. Only 71 of the plates yielded *Br. abortus* and in no case did the guinea pig prove negative where the plate inoculated with the same material was positive. However, in another series of 36 samples in which the guinea pigs were injected approximately 24 hours after the plates were made, *Br. abortus* was obtained by the plate method in 5 samples which were negative by guinea-pig injection. Five other samples were proved positive by guinea-pig inoculation, but failed to show *Br. abortus* colonies on the plates. The number of positive samples by each method was 17 and the negative 19.

As considerably more time and care was taken, both in the preparation of the medium used and in the examination of suspicious colonies, than is feasible for ordinary routine examination, it seems probable that failure in at least 25–30 per cent of the cases must be expected when the culture method alone is depended upon.

In addition to the failure of the culture medium to detect *Brucella abortus* in some materials which may be proved to be positive by guinea-pig inoculation, the occasional occurrence of dye-tolerant contaminants in large numbers in some suspected materials may be a source of error, or of considerable annoyance. When contamination by organisms with colonial appearance resembling that of *Br. abortus* occurs, the time and labor involved in ascertaining the identity of the organisms soon approaches that necessary for guinea-pig inoculation.

The ever present danger that a given lot of medium may be unsatisfactory for the growth of *Brucella abortus,* because of unsuitable H^+ concentration, poor physical characteristics, etc., also adds to the disadvantages of this method. This can only be avoided by testing each lot with cultures of *Br. abortus,* which takes several days and allows the medium to become somewhat dry.

While the culture method, in our opinion, cannot replace animal inoculation, it would seem to have distinct value as a supplement to the slower method. By the use of both methods, those animals inoculated with materials which prove to be definitely positive by the culture method may be destroyed without examination, and the final decision on materials which give negative or unsatisfactory results on the plates may be reserved until the guinea pigs are autopsied. Or, in order to avoid the injection of a large number of guinea pigs, milk may be preserved with 1 per cent boric acid or held at ice-box temperature until the results of the cultures have been obtained. Those samples which prove negative may then be injected into guinea pigs.

GUINEA-PIG INJECTIONS

Selection of Animals.—In this laboratory male guinea pigs weighing from 350 to 400 grams have been found to be the most satisfactory test animals. An animal of less than 300 grams is apt to show ill effects from the large quantities of inoculum, and from the handling necessary at the time of inoculation. Male pigs are preferred because of the characteristic *Brucella abortus* lesions produced in the testicles, and because they seem somewhat more suspectible to infection with small numbers of organisms. Table 1, although it includes only a small number of tests, indicates the relative susceptibility of male and female guinea pigs when inoculated with naturally infected milk.

The number of organisms per gram of spleen, as given in table 1, was estimated by the method described by Hagan.[12] This consists in grinding a weighed portion of the infected spleen and making

dilutions from which plates are seeded. The simple but ingenious apparatus which Hagan described for the grinding of the spleen is made by selecting two common test tubes, one sufficiently smaller than the other to allow it to be inserted within the first. The pestle tube should fit into the outer one rather loosely to avoid having the emulsion creep up between the tubes. The tubes may be fitted together, weighed, capped with paper, and sterilized. When ready for use, a portion of the spleen is placed in the larger tube, and after replacing the inner tube, the weight of the spleen is determined. After grinding,

TABLE 1

COMPARISON OF POST-INOCULATION PERIODS AND THE RELATIVE SUSCEPTIBILITY OF MALE AND FEMALE GUINEA PIGS TO BRUCELLA ABORTUS IN MILK

Milk from Cow No.	Killed at	Sex	Weight of spleen in grams	*Br. abortus* per gram spleen	Agglutinin titre (1–25, 1–50, etc.)	Lesions
A652	4 weeks	♂	1.555	6,850	+±−−−−−−−−	−
		♀	1.237	.....	−−−−−−−−−−	−
	6 weeks	♂	4.175	578,400	+++++−−−−−	+
		♀	1.300	.....	−−−−−−−−−−	+
	8 weeks	♂	3.035	147,800	+++++++++−	+
		♀	1.210	446,300	+++−−−−−−−	Slight
A656	4 weeks	♂	0.802	15,000	+++±∓−−−−	+
		♀	0.945	.....	∓−−−−−−−−	Slight
	6 weeks	♂	0.845	.....	∓−−−−−−−−	−
		♀	1.095	.....	−−−−−−−−−	−
	8 weeks	♂	0.645	307,700	+++++±−−−	Very slight
		♀	1.025	204,700	+++∓−−−−−	Very slight
A659	4 weeks	♂	0.595	9,950	+++−−−−−−	−
		♀	1.100	8,100	+++±−−−−−	+
	6 weeks	♂	1.020	68,600	+++±−−−−−	+
		♀	1.285	700	++±−−−−−−	Slight
	8 weeks	♂	5.440	134,700	++++++++++	+
		♀	3.005	40,800	+++++++++±	+

the spleen may be diluted with sterile saline. A convenient initial dilution is 10 cc per gram of spleen.

Testing of Guinea Pigs.—For several years it was the practice in this laboratory to bleed all guinea pigs a few days previous to inoculation, and to test the blood for *Brucella abortus* agglutinins. This was discontinued, but later two animals, inoculated with materials unlikely to have contained *Br. abortus* and kept in the laboratory for several months, became infected with *Br. abortus*. Since that time all guinea pigs used for *Br. abortus* experiments have been tested before inoculation. All animals mentioned in this paper were so tested. However, the results of several thousand tests are now on record, and in no case has an animal shown even a trace of agglutinin content

in its blood. Because of these continuously negative results it seems unlikely that guinea pigs brought into the laboratory within a few weeks previous to inoculation will be found to be infected with *Br. abortus*. Animals which have been raised or held for a considerable length of time in a laboratory where *Br. abortus* infected animals are kept should be tested before being used.

Blood may be drawn from the heart for testing, but animals treated in this manner should be kept several days, subsequent to the bleeding, before inoculation. The following method, a modification of that described by Seddon,[17] has the advantage of not affecting the animal to any appreciable extent.

Ordinary agglutination test tubes are marked by means of a file at points indicating 4.5 cc and 5 cc. The tube is filled to the 4.5 cc mark with 0.85 per cent salt solution containing 1.00 per cent sodium citrate and 0.50 per cent phenol. An ear vein of the test animal is cut and the edge of the tube is rubbed repeatedly upward over the cut to collect the slowly oozing blood until the mixtures reaches the 5 cc mark. The guinea pig is then ear-tagged and the tag number placed on the cork of the test tube.

After standing a short time, the corpuscles settle sufficiently so that the clear liquid, representing a serum dilution of approximately 1–20, may be drawn off, and the agglutination test performed. Blood may also be collected from the ear and allowed to clot in a capillary tube. Sufficient serum may be obtained in this manner to perform the agglutination test.

Housing to Prevent Accidental Infection.—During the course of an experiment in which large numbers of guinea pigs were inoculated with materials containing *Brucella abortus,* circumstantial evidence seemed to indicate that, in a very small percentage of cases, guinea pigs inoculated with materials free from *Br. abortus* became infected by contact with diseased pigs. In light of Surface's[20] report of a spontaneous outbreak of *Br. abortus* infection among guinea pigs, it was decided to keep each inoculated animal in a separate cage. For this purpose ordinary cracker tins 10½ by 10½ by 8 inches, with hinged tops, have been found very satisfactory after the top has been perforated with ½-inch holes.

Although the possibility of infection by contact is present, it apparently does not occur readily. Hagan[12] was unable to infect animals by keeping them in close contact with animals of the same sex. He did, however, in one case succeed in producing infection in a female guinea pig which was kept with an infected male. In table 2 the results of a similar test, in which we also were unsuccessful in the attempt to transmit the disease by contact, are given.

TABLE 2

RESULTS OF POST-MORTEM EXAMINATIONS AND CULTURES FROM GUINEA PIGS IN CONTACT FOR 6 WEEKS WITH GUINEA PIGS INFECTED WITH BRUCELLA ABORTUS

Cage	Treatment	Guinea pig No.	Sex	Blood serum*	Lesions	Spleen culture	Urine culture
	Inoculated†	7222	♂	Died; no test	+	+	+
		7223	♂	++++	+	+	+
		7224	♂	++++	+	+	+
	Not inoculated						
A	Ear tagged	7225	♂	----	−	−	
		7226	♂	----	−	−	
	Ear tagged; skin of abdomen abraded weekly	7227	♂	----	−	−	
		7228	♂	----	−	−	
	No ear tags; no visible abrasions	7256	♀	----	−	−	
		7257	♀	----	−	−	
	Inoculated†	7229	♀	++++	+	+	+
		7230	♀	++++	±	+	+
		7231	♀	++++	+	+	−
	Not inoculated						
B	Ear tagged	7232	♀	----	−	−	
		7233	♀	----	−	−	
	Ear tagged; skin of abdomen abraded weekly	7234	♀	----	−	−	
		7235	♀	----	−	−	
	No ear tags; no visible abrasions	7254	♀	----	−	−	
		7255	♀	----	−	−	
	Inoculated†	7258	♀	Died	+	+	+
		7259	♀	Died			
		7260	♀	++++	+	+	
		7261	♀	++++	+	+	+
	Not inoculated						
C	Ear tagged	7262	♀	----	−	−	
		7263	♀	----	−	−	
	Ear tagged; abdomen shaved and abraded weekly	7264	♀	----	−	−	
		7265	♀	----	−	−	
	No ear tags; no visible abrasions	7266	♀	----	−	−	
		7267	♀	----	−	−	
	Inoculated†	7268	♂	++++	+	+	+
		7269	♂	++++	+	+	+
		7270	♂	Died	+	+	+
	Not inoculated						
D	Ear tagged	7271	♂	----	−	−	
		7272	♂	----	−	−	
	Ear tagged; abdomen shaved and abraded weekly	7273	♂	----	−	−	
		7274	♂	----	−	−	
	No ear tags; no visible abrasions	7275	♂	Died	−	−	
		7276	♂	----	−	−	

* The titre limit of these serums was not determined.

† Inoculated intraperitoneally with 0.5 cc of a suspension of *Br. abortus* No. 80.

PREPARATION OF MATERIAL FOR GUINEA-PIG INJECTION

Milk.—The isolation of *Brucella abortus* from the milk of cows by guinea-pig inoculation was first reported by Schroeder and Cotton[16] and Smith and Fabyan[18] in 1911, and has since been used extensively for the detection of shedder cows in infected herds. It is of importance also in cases of human infection attributed to infected milk; for a positive agglutination test of the blood serum of the cow supplying the milk does not necessarily indicate the presence of the organism in the milk.

TABLE 3

COMPARISON OF THE NUMBER OF BRUCELLA ABORTUS ORGANISMS FOUND IN CREAM AND SEDIMENT OBTAINED BY CENTRIFUGING AND BY GRAVITY

Sample No.	Centrifuged cream and sediment: organisms per cc	Cream and sediment obtained by standing: organisms per cc
1	10,960	2,650
2	1,960	1,060
3	3,750	480
4	140	0
5	3,090	70
6	2,290	510
7	240	100
8	900	1,720
9	1,100	370
10	1,370	130
11	7,080	350
12	170	0
13	520	10
14	440	0

Evans,[7] Carpenter,[5] and others have shown that the number of *Brucella abortus* organisms found in milk is relatively small. For this reason, the direct inoculation of the milk may fail to produce disease in the guinea pigs. Concentration of the bacteria by centrifuging may yield positive results in cases where inoculation of the whole milk would fail to reveal the organism. The method outlined below has been in use in this laboratory for several years, and previous to that time was used in the United States Department of Agriculture Bureau of Animal Industry laboratories in Washington. It has also been outlined by Carpenter. The milk is collected into a sterile, wide-mouthed bottle with aseptic precautions, iced, brought to the labora-

tory, and from 70 to 90 cc of it is transferred to 100 cc sterile centrifuge tubes. After the milk has been centrifuged at high speed for 20 minutes, most of the skimmed milk is discarded by decanting, using a sterile glass rod to prevent the disk of cream from leaving the tube. Two to 4 cc of the skimmed milk, together with sediment and cream, are retained, and the whole thoroughly mixed with the rod. About 3 cc of this concentrate is inoculated intraperitoneally into the guinea pig. A part of the skimmed milk may conveniently be saved, and, after the addition of rennet, be used to determine the agglutinin content of the milk.

If a centrifuge is not available, the milk may be kept overnight on ice, and the cream and sediment mixed in the same manner as in the centrifuged samples. However, comparative tests show that this method is not so efficient as the centrifuge method, as indicated in table 3. This is, however, contrary to the findings of Huddleson, Hasley, and Torrey,[13] who gave the following:

	Cow 8	Cow 84
	Br. abortus organisms	*Br. abortus* organisms
Centrifuged cream 0.1 cc	207	77
Gravity cream 0.1 cc	552	209

For milk which must be shipped without ice, Gilbert, Coleman, and Groesbeck[10] have proposed the use of 30 per cent glycerine as a preservative. Traum and Henry[21] have found that naturally infected milk kept at room temperature for more than 10 days will still induce *Brucella abortus* infection in guinea pigs when inoculated intraperitoneally, if 1 per cent boric acid is added to the milk at the time it is drawn.

Udder Exudate.—In making a survey of the extent of *Brucella abortus* infection in a dairy herd it is often very desirable to include the dry cows as well as those in active lactation. For several years excellent results have been obtained in this laboratory by the inoculation of the semigelatinous exudate obtained from the udders of dry cows. The information obtained in this manner is especially important in an epidemiological or epizoological study where only a single test of the herd is possible.

Fetal Tissues.—The isolation of *Brucella abortus* from aborted fetuses which are in good condition may usually be accomplished by the use of culture media without recourse to guinea-pig inoculations. However, badly torn or poorly preserved specimens may contain so many contaminating organisms that direct cultures will be badly overgrown. In such cases, small fragments of tissue may be ground in a mortar, suspended in salt solution, and inoculated. If the material

is heavily contaminated, gentian violet in a final dilution of 1 to 200,000 may be added to the suspension and allowed to stand 30 minutes, in order to reduce the number of contaminating organisms and prevent peritonitis in the guinea pig. For ordinary routine examination, the stomach and rectal contents of the fetus are combined and inoculated into one guinea pig, and pieces of the lung and liver are inoculated into another pig.

Vaginal Swabs.—When the placental and fetal materials from an aborting animal are not obtainable, the organism may in some cases be recovered by the inoculation of material obtained by means of a vaginal swab. For such inoculations a convenient swab may be pre-

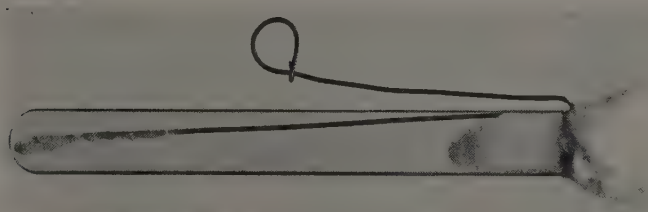

Fig. 1. Sterile swab for obtaining uterine exudate from vagina or uterus of cattle.

pared by wrapping cotton on a stiff wire about 15 inches long. The swab end of the wire is inserted into a large (1 by 8 inch) test tube, the tube being plugged with cotton and the portion of the wire remaining outside the tube bent down against the tube to form a compact package (fig. 1). The whole is wrapped in paper and sterilized. When the charged swab is received in the laboratory, it is removed from the tube and placed in a tube containing 3 or 4 cc of sterile saline solution. After being soaked for 10 to 15 minutes and sufficiently shaken to insure suspension of the material, the salt solution is inoculated into the guinea pig.

Urine.—The sediment obtained by the centrifugalization of urine may be inoculated in the same manner as is milk. In the case of bovine material this procedure probably has little if any value, as several attempts to isolate *Brucella abortus* from the urine of positive cows have uniformly failed.

Guinea-pig inoculations and cultures have been made from the urine of 13 cows, most of which were strongly positive to the agglutination test for *Brucella abortus*. The bladders were secured at the time of slaughter, and 50 to 100 cc of urine centrifuged. The sediment and the film which appeared on the surface of the centrifuged

urine were inoculated into the peritoneal cavity of guinea pigs, and cooked-blood-agar plates were planted. In all cases the cultures and guinea pigs were negative for *Br. abortus*.

The inoculation of urine from human or caprine sources may give positive results. The occurrence of the *Brucella* organism in the urine of artificially inoculated guinea pigs has been reported several times, and will be dealt with later in this paper.

Blood.—Citrated or defibrinated blood may be inoculated into the peritoneal cavity of guinea pigs. Blood which has been allowed to clot is thoroughly ground in a sterile mortar, suspended in salt solution, and inoculated. The method devised by Boez and Robin[4] for successful blood cultures of various organisms might well be used for animal inoculation, as well as for culture seeding, in the attempted isolation of *Brucella abortus*. By the use of acid potassium sulfate to reduce the pH of the blood at the time of collection, the bactericidal action is destroyed.

Briefly, the technique as outlined by Boez and Robin is as follows: The blood is drawn into a sterile tube containing 2 cc of the following solution:

Sodium citrate	2.75 grams
Potassium acid sulfate	5.60 grams
Distilled water to make	100 cc

The pH of the blood after being citrated with the above solution is about 5.5, sufficiently low, according to Boez and Robin, to destroy the bactericidal property of the blood. Ten cc of the citrated blood is added to 100 cc of glucose broth, which has a pH 8.3. The final reaction will be found to be about pH 7.5.

Soule[19] has published the following technique: Cattle blood was cultured for *Brucella abortus* by adding 50 cc samples to 450 volumes of sterile glycerol infusion broth and subsequently incubating in air enriched with CO_2. The blood was diluted as soon as withdrawn and was not chemically treated or defibrinated to prevent clotting. At 2–4 day intervals, samples were removed and streaked on the surface of glycerol agar. He reports tests on over 5,000 ordinary herd-run cows. In the first series of tests agglutinins were present in the blood stream of 2,237 of the animals but only 299 gave positive blood cultures. In the second series agglutinins were present in 2,607 cases with 206 positive blood cultures. There were 40 positive blood cultures reported without concomitant blood agglutinins.

Feces.—Barger and Hayes[3] were able to demonstrate the presence of *Brucella abortus* in the feces of calves which had ingested

infected material, by diluting about 5 grams of fecal material with 20 cc of sterile salt solution. About 2 cc of the resulting suspension was inoculated intraperiatoneally into guinea pigs.

Amoss and Poston[1, 2] more recently have outlined a method by which the *Brucella* organisms in human feces may be concentrated by use of strong *Br. abortus* antiserum, and isolated on eosin-methylene-blue plates.

METHODS OF INJECTION

While the subcutaneous inoculation of guinea pigs with suspected material is the most generally used method for the detection of *Brucella abortus,* intraperitoneal inoculation has been used in this laboratory with considerable success. There are advantages and disadvantages connected with both methods. The test animals are better able to overcome a large number of contaminating organisms when the inoculation is subcutaneous. In the case of milk which contains large numbers of streptococci, this is of considerable importance. However, milk or other material sufficiently contaminated to cause the death of the guinea pig when inoculated intraperitoneally is a very poor sample to use for the detection of *Br. abortus* by any method. Carpenter and Boak[6] have shown that the *Br. abortus* organisms are no longer viable in cream when the acidity reaches pH 5.0 or lower. Badly contaminated milk samples are usually below this figure.

In the routine examination of the individual cows in a large dairy herd during the past two years, approximately 1,300 guinea pigs have been inoculated intraperitoneally with milk. The samples were usually inoculated about 8 hours after being drawn, but not infrequently 24 hours elapsed between collection and inoculation. In this group, 37 pigs succumbed to peritonitis due, probably, to the inoculum. Of these animals, 17 were in one lot of 30 which received milk brought to the laboratory during very warm weather, without ice. It is doubtful if this later group would have become infected with *Brucella abortus* even though the organism had been present at the time the samples were collected. The loss from intraperitoneal inoculation of properly collected and handled materials is relatively unimportant. An advantage which this method has over subcutaneous inoculation is the possibility of inoculating comparatively large quantities of material; 3 cc or more may be given intraperitoneally with no ill effect, whereas such amounts given subcutaneously at a single point of injection cause acute discomfort to the animal. This is of particular importance in the inoculation of milk or other materials containing few *Br. abortus* organisms.

OPTIMUM TIME FOR AUTOPSY

The procedure which probably varies most widely in the different laboratories is the length of time which is allowed to elapse between inoculation and autopsy. This period varies from one week to two or more months. Nelson[15] has reported a method by which *Brucella abortus* may be recovered from the guinea pig 5 days after inoculation. Carpenter[5] keeps the pigs for from 4 to 5 weeks and Gilbert, Coleman, and Groesbeck[10] in a report on some comparative tests, reached the conclusion that guinea pigs inoculated with material suspected of containing *Br. abortus* should not be killed before the end of the the fifth week. These tests were made with two lots of animals, one killed between the fourth and fifth week, and the other at about the end of the eighth week, but no data were given as to the advantages or disadvantages of slaughter at any interval between these two periods.

In this laboratory it has been the custom to adhere rather rigidly to a period of from 40 to 44 days between inoculation and slaughter. This interval has given consistently good results, and the correlation of agglutinins, macroscopic lesions, and cultures has been rather close in the majority of cases.

A small series of comparative tests, the results of which are shown in table 1, would seem to indicate that a period of 8 weeks is slightly superior to one of 6 weeks. However, the maintenance of a large number of inoculated pigs for 2 additional weeks has many disadvantages, and the number of positive cases obtained by the additional time is probably rather small. In cases of particular interest or importance, the inoculation of two animals with the same material is very desirable because of the possibility of individual resistance of the test animal and of loss by intercurrent diseases. When two animals are inoculated, one may be slaughtered at the end of 6 weeks and the other held for 8 weeks in case the first is negative.

AUTOPSY TECHNIQUE

The technique outlined below is given in detail because in this laboratory it has been found to be a simple, convenient, and successful method which can be used for making post-mortem examinations of large numbers of guinea pigs. As will be shown later, the presence of *Brucella abortus* in the spleen of an inoculated animal is frequently the only indication of infection. For this reason this technique of

autopsy has been developed with the primary object of procuring a culture from this organ free from contamination.

The guinea pig to be autopsied is placed in a Novy jar containing chloroform and left until respiration has ceased. In order to obtain blood for the agglutination test, it is preferable not to allow the animal to remain in the chloroform until the blood begins to clot. After the animal has been dipped in a weak solution of liquor cresolis compositus, it is fastened to a Carpenter[5] autopsy board (fig. 2) and an incision is made in the skin from the throat to a point somewhat posterior to the sternum, along the median ventral line. Another cut is made from the throat to near the right foot, and the

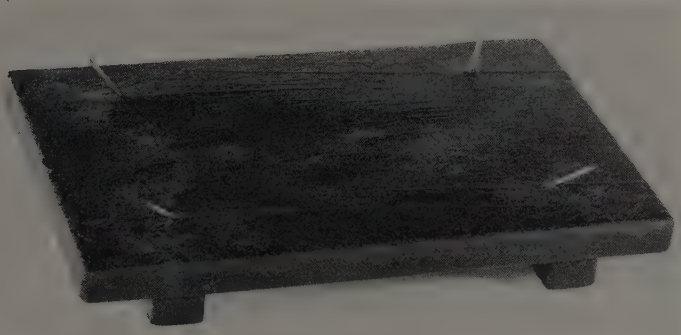

Fig. 2. Carpenter autopsy board for the post-mortem examination of guinea pigs.

triangular flap of skin thus formed is freed from the body wall, exposing the right axillary region and forming a pocket in which blood may collect when the axillary vessels are severed.

When the blood has been transferred to a tube, the skinning is completed and loose hair is destroyed by passing the flame of an inverted Bunsen burner several times over the entire body. By means of a small soldering iron the surface of the left side of the abdomen is thoroughly seared. The board is then placed in an inclined position by elevating and propping the side to the operator's right. With flamed scissors and forceps, a roughly semicircular incision is made in the abdominal wall, starting about 1 inch anterior to the anus, bearing to the right and about 1½ inches from the median line, and crossing back over and beyond the median line at the tip of the sternum. The resulting flap of tissue is folded over to the left, and the intestines may be grasped with forceps and pulled toward the

left. By this means, because of the inclined position of the board, the stomach and intestines are dislodged and the spleen exposed in a position convenient for examination and culture. The tip of the spleen is grasped with flamed forceps held in the left hand, while scissors are used to free the spleen. The tissue is transferred to a cooked-blood-agar slant by means of a stiff chrome-wire loop. It is desirable to obtain as large a piece of tissue as possible for seeding. In the case of a normal-sized spleen, the entire organ is used except for a small piece at the tip which is clipped off in order to produce a rupture in the capsule. In the case of enlarged spleens, a piece of tissue approximately equal to a normal spleen is used.

After the culture has been made, the thoracic cavity may be opened and the animal examined for other lesions of brucelliasis.

All spleen cultures are incubated in 10 per cent carbon dioxide for 4 to 6 days; then the slants are examined and if no growth is visible, the spleen tissue is crushed and spread over the surface of the slant with a stiff wire loop. Frequently after further incubation, cultures are obtained by this method which would be overlooked if the tissue had been left undisturbed. After about 21 days of incubation, tubes showing no growth are discarded.

Transplants are made from all cultures and the resulting growth is suspended and used as agglutinating fluid and tested with known positive and negative *Brucella abortus* antiserum.

OBSERVATIONS ON THREE CRITERIA OF INFECTION IN GUINEA PIGS

Agglutinin Production in Infected Guinea Pigs.—Little correlation is found between the number of organisms inoculated and the agglutinin titre in the blood of guinea pigs at the end of 6 weeks. This refers only to animals inoculated with naturally infected milk, the bacterial count of which has been determined by counts on gentian-violet cooked-blood agar. The blood-serum titre of the infected animals, at the time of slaughter, varies from negative at a dilution of 1 to 25 to positive at 1 to 6,400, with the majority positive within the range between dilutions of 1 to 400 and those of 1 to 1,600. The number of organisms found in naturally infected milk is relatively small, and the number inoculated in 2 cc of cream and sediment rarely exceeds 10,000.

Extensive lesions, typical of brucelliasis in a test animal, are usually accompanied by demonstrable agglutinins in the blood serum, but the extent of the lesions is no indication as to the titre of the *Brucella* agglutinins. Occasionally slight but definite lesions are

found in animals whose blood serum fails to cause agglutination of *Br. abortus* antigen even at a dilution of 1 to 25. A number of animals have been encountered with very extensive lesions whose blood serum had a titre of only 1 to 50 or 1 to 100.

In an appreciable percentage of cases, guinea pigs will be found whose blood serum is negative at a dilution of 1 to 25 but whose spleen yields *Brucella abortus* when cultured. These animals may show slight macroscopic lesions, but are usually normal in appearance. In compiling the results of 1,214 guinea pigs inoculated with suspected material, it was found that 438 cultures of *Br. abortus* were obtained from the spleens. Of this number, 28, or slightly over 6 per cent, were obtained from guinea pigs whose blood showed no agglutination at a dilution of 1 to 25 or over.

In the light of these findings, the rather widespread practice of keeping test animals for an indefinite time and eventually discarding them as negative if the agglutination test performed with their blood is repeatedly negative, seems open to considerable objection. How many of the 28 guinea pigs mentioned above would have eventually exhibited agglutinins, it is of course impossible to determine, but it seems probable that some of them would have been able to overcome the infection without any agglutinin production. Still other animals may be physiologically unable to produce antibodies regardless of the course of infection, and therefore would be included in the negative group if agglutinin production alone were depended upon. Nonvirulent variants of *Br. abortus* which produce no agglutinins have also been isolated from the spleens of guinea pigs at least 9 weeks after inoculation.

Of 427 animals with an agglutinin titre of 1 to 25 or over, cultures of *Brucella abortus* were not obtained from 17, or 4 per cent. Eight of these failures were due to contamination and we feel that the remainder could be explained by some failure in technique. In view of these results, it seems highly probable that *Brucella abortus* can be isolated from the spleen of any guinea pig whose agglutinin titre for this organism is 1 to 25 or higher, if the culture is made 6 weeks after inoculation.

Lesions in Infected Guinea Pigs.—In the routine examination of guinea pigs for lesions of brucelliasis 6 weeks after inoculation, organs which will be found to be most frequently involved are the spleen, the liver, the male genital organs, the precrural, sublumbar, and inguinal lymph nodes, the lungs, and the leg joints. For a detailed and complete description of lesions, the reader is referred to the works of Fabyan,[8] Schroeder and Cotton,[16] or Jaffe.[14] However, it must be borne in mind that the lesions described by these authors

are, in most cases, the results of infections with massive doses followed by an interval of several months before autopsy, and comparable lesions are not to be expected when test animals are slaughtered 6 weeks after inoculation.

Macroscopically the spleen may be enlarged slightly or may be enlarged up to six times its normal size. The surface is usually nodular (fig. 3), but in cases of an acute nature, the spleen may be

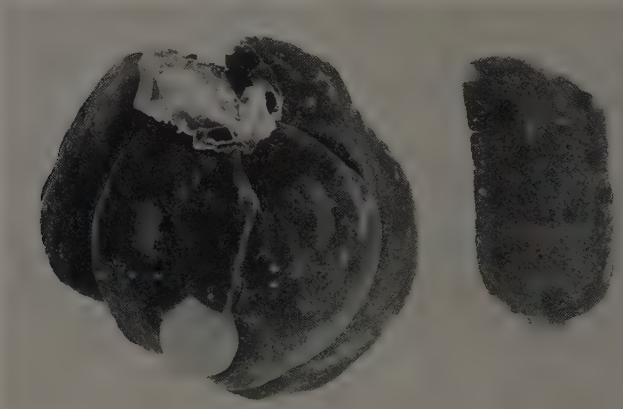

Fig. 3. Liver and spleen of guinea pig killed 6 weeks after intraperitoneal inoculation with milk containing *Brucella abortus*.

smooth. The nodules in the early stages of their development are hemorrhagic, later becoming encapsulated, grey, discrete, and may have a necrotic center.

Just beneath the capsule on the surface of the liver, small grey glistening nodules may usually be found. These nodules range in size from 0.5 to 2.0 mm in diameter, are discrete, and may or may not have an opaque center. In exceptional cases the entire liver may be studded with the nodules, but usually from 10 to 50 occur (fig. 3).

Macroscopic lesions in the female genital organs are so rare that they are of no importance for routine diagnosis. The reverse is true, however, in the case of the male organs. In most cases of intraperitoneal inoculation of male guinea pigs, lesions may be found in the testicles proper, in the epididymis, or in the walls of the sac surrounding the organ. Adhesions of the testicles or epididymis to the sac are frequent. Abscesses containing creamy, yellow, or white pus are occasionally found in the testicle proper (fig. 4), but more frequently in the epididymis (fig. 5). The tubules of the epididymis

become enlarged and may be disintegrated sufficiently so that they do not appear sharp in outline as do the tubes in a normal organ. Abscess formation may occasionally be found in the cremaster muscles with no macroscopic change in the epididymus. Atrophy of one or both testicles also occurs, with or without abscess formation.

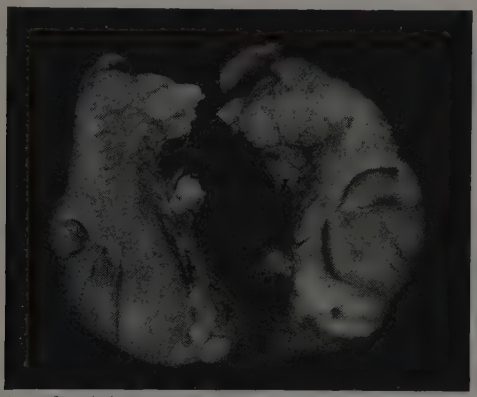

Fig. 4. Testes of guinea pig killed 6 weeks after intraperitoneal inoculation with milk containing *Brucella abortus*. Note the abscess formation in the distal portions of the testes proper.

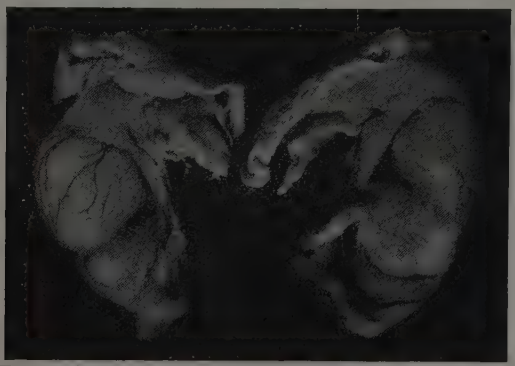

Fig. 5. Testes of guinea pig killed 6 weeks after intraperitoneal inoculation with milk containing *Brucella abortus*. Note abscesses in epididymes.

Noticeable enlargement of the sublumbar lymph nodes almost invariably accompanies testicular involvment. This increase in size usually varies from two to six times normal. The nodes are often hyperemic and occasionally definitely hemorrhagic. Abscess formation is rather rare in the lymph nodes of animals that are inoculated with small numbers of bovine *Brucella abortus* and kept only 6 weeks

before slaughter. The precrural and inguinal lymph nodes often show alteration similar to that found in the sublumbars. Macroscopic changes in other lymph nodes of the body are relatively rare, although occasionally enlargement of axillary, mesenteric, and bronchial nodes is noted.

Fig. 6. Joint lesions in guinea pigs, produced by the injection of massive doses of *Brucella abortus*.

In a rather small percentage of cases, clear glassy irregularly shaped areas are found just beneath the pleura. These vary from 1 to 5 mm in diameter. They usually show an opaque center. When the lesions in the lungs are large or numerous, some enlargement of the bronchial lymph nodes occurs.

Lesions of the joints are extremely rare in animals inoculated with bovine tissues and materials, and slaughtered at the end of 6 weeks. With massive infecting doses such as cultures or infected utero-chorionic exudate, swollen carpel and tarsal joints occur rather frequently (fig. 6).

In addition to the previously mentioned manifestations, other less common lesions may from time to time be encountered. In this group would be included an abscess 1 cm in diameter filled with creamy pus, on the wall of the urinary bladder (fig. 7), which upon culture yielded only *Brucella abortus;* subcutaneous or intramuscular ab-

scesses in the abdominal wall at the point of inoculation which may be caused by *Br. abortus;* and a large subcutaneous abscess behind the ear, open to the outside, which also yielded *Br. abortus.*

In the examination of large numbers of guinea pigs for lesions of brucelliasis. it will be found that those outlined above may appear singly or in almost any combination; however, the spleen, liver, and

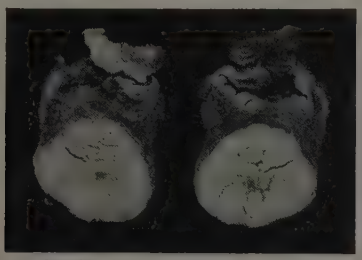

Fig. 7. Abscess on wall of urinary bladder of a guinea p'g. This animal had been inoculated with milk 6 weeks previous to the time of autopsy.

testicle lesions are ordinarily rather uniform as to extent and occurrence. The liver lesions are the most characteristic of all the lesions described. It is rare indeed to find typical small grey translucent nodules on the surface of the liver without finding *Brucella abortus* agglutinins in the blood or obtaining *Br. abortus* in cultures made from the spleen. On the other hand, the absence of liver lesions is not necessarily indicative of freedom from *Brucella* infection.

The relation of lesions to agglutinins and to positive cultures is rather close. However, in a total of 516 guinea pigs which showed

TABLE 4

Occurrence of Agglutinins, Lesions, and Positive Spleen Cultures in Guinea Pigs Inoculated with Milk Naturally Infected with Brucella Abortus and Slaughtered at the End of 6 Weeks

Agglutinins in blood	Lesions	Cultures	Number of animals	Percentage of total
+	+	+	427	82.7
−	+	+	5	1.0
+	−	+	56	10.8
−	−	+	22	4.3
+	+	−	1	0.2
−	+	−	4*	0.8
+	−	−	1	0.2
Total animals			516	100 0

* These guinea pigs showed lesions which were macroscopically indistinguishable from those produced by infection with *Brucella abortus*, but which might have been due to other causes.

evidence of infection, the correlation shown in table 4 was observed. From this table it will also be found that 98.8 per cent yielded positive spleen cultures, 93.8 per cent showed agglutinins in their blood at a dilution of 1 to 25 or over, and 84.7 per cent showed macroscopic lesions.

Cultures from Infected Guinea Pigs.—

1. Spleen cultures: Most workers have found the spleen to be the best organ for use in obtaining cultures of *Brucella abortus.* Observations in this laboratory in the past four years on some 3,000 guinea pigs inoculated with materials suspected of containing *Br. abortus* have amply borne out this conclusion. As stated above, it rarely occurs that one fails to obtain a culture from the spleen of an animal that manifests any other indication of infection. This holds true for guinea pigs whose spleens show no macroscopic lesions, as well as for those whose spleens show gross pathological changes.

2. Urine cultures: In order to determine the occurrence of *Brucella abortus* in the blood and urine of infected guinea pigs, a series of cultures were made from the heart blood and from the urine of animals showing macroscopic lesions of brucelliasis.

The cultures from the urinary bladder were made by thrusting a Pasteur pipette through the wall after the bladder had been thoroughly seared to avoid contamination with *Brucella abortus* from the peritoneal cavity. One-half to 2 cc was obtained in most cases, but only a drop or two in a few instances. The urine was transferred to cooked-blood-agar slants and the slant tipped once or twice in order to insure distribution over the surface. The cultures were incubated in 10 per cent carbon dioxide.

Cultures were made from a total of 106 infected animals and *Brucella abortus* was recovered from 24, or 22.6 per cent of these. Seventy-nine of the cultures showed no growth, 2 gave pure cultures of a *Streptococcus,* and 1 was overgrown with an air contaminant. Spleen cultures were obtained from all these animals.

The difference between the percentage of positive urine cultures in the males and in the females was marked. The sex was recorded in only 93 of the 106 animals whose urine was cultured. Of these, 50 were females and 43 were males. Five of the females (10 per cent) yielded positive cultures, whereas 15 (34.8 per cent) of the urine cultures from the males were positive. This difference is undoubtedly due to the frequency with which the male genital organs are infected, and it may be assumed that in many cases the organisms are carried to the bladder from broken-down testicular lesion. However, one case (fig. 7) has been observed of a definite infection in the bladder wall.

3. Blood cultures: Heart-blood cultures were made in a manner similar to that employed for securing urine cultures. In the case of blood cultures, however, it is important to stir the clotted blood 4 to 6 days after the initial seeding. A considerable number of the cultures which proved positive after the thorough stirring would have been discarded as sterile if the clot had not been broken up. Close inspection of the tube is necessary to avoid discarding cultures containing a small amount of growth. Several cases were encountered in which the only evidence of growth was a thin grey film between the tube and the butt of the slant. While most of the positive cultures showed very few colonies, several were encountered in which, before stirring, the surface of the slant was completely studded with colonies.

In cultures made from the heart blood of 126 infected guinea pigs, *Brucella abortus* was recovered from 60, or 47.6 per cent. There was no significant difference between the number of positive cultures obtained from the male and the female guinea pigs. Of the 117 animals whose sex was recorded, 66 were female and 51 were male. Positive cultures were obtained from 33, or 50 per cent, of the females, and from 24, or 47 per cent, of the males.

SUMMARY

A comparison of direct-culture methods with guinea-pig inoculations for the isolation of *Brucella abortus* from milk has been made. Guinea-pig inoculations have proved much more efficient.

The technique for the isolation of *Brucella abortus* by guinea-pig inoculation and by culture methods is given in detail.

An attempt has been made to determine the reliability of the three common criteria of infection in artificially inoculated guinea pigs; namely, agglutinin production, lesions, and spleen cultures. The relation of positive spleen cultures, agglutination titers over 1 to 25, and macroscopic lesions, in a total of 516 guinea pigs showing some evidence of infection, was 98.8 per cent, 93.8 per cent, and 84.7 per cent, respectively. For this reason the spleen-culture indication is considered the most reliable of the three.

The incidence of *Brucella abortus* in the urine and blood of guinea pigs 6 weeks after inoculation with infected material has been observed. This organism was recovered in cultures of the urine in 22.6 per cent of 106 guinea pigs known to be infected, and the frequency of the organism in the urine of males was over three times that in the urine of females. *Br. abortus* was also obtained in cultures from the blood in 47.6 per cent of 126 guinea pigs known to be infected. There was no significant difference between the number of positive blood cultures obtained from the male and the female guinea pigs.

LITERATURE CITED

[1] Amoss, Harold L., and Mary A. Poston.
1929. Undulant (Malta) fever. Isolation of the *Brucella* organism from the stools. Jour. Amer. Med. Assoc. 93:170–171.

[2] Amoss, Harold L., and Mary A. Poston.
1930. Cultivation of *Brucella* from the stools and bile. Jour. Amer. Med. Assoc. 95:472–483.

[3] Barger, E. H., and F. M. Hayes.
1924. The discharge of *Bacterium abortum* in the feces of calves fed milk containing the organism. Jour. Amer. Vet. Med. Assoc. N. S. 19:328–336.

[4] Boez, L., and L. A. Robin.
1929. Sur la destruction du pouvoir bactéricide du sang. Application a l'hemoculture. Comptes Rendus de la Soc. Biol. (Paris) 101: 1009–1012.

[5] Carpenter, C. M.
1921. The bacteriology of the female reproductive organs of cattle and its relation to the diseases of calves. New York State Vet. Coll., Cornell Univ. Ann. Rept. 1920–21:67–107.

[6] Carpenter, C. M., and Ruth Boak.
1928. *Brucella abortus* in milk and dairy products. Amer. Jour. Pub. Health 18:743–751.

[7] Evans, Alice C.
1918. *Bacterium abortus* and related bacteria in cows' milk. Jour. Infect. Diseases 23:354–372.

[8] Fabyan, M.
1912. A contribution to the pathogenesis of *B. abortus*, Bang II. Jour. Med. Research 26:441–488.

[9] Fleming, Alexander.
1919. On some simply prepared culture media for *B. influenza* with a note regarding the agglutination reactions of sera from patients suffering from influenza to this bacillus. Lancet 196:138–139.

[10] Gilbert, Ruth, M. B. Coleman, and W. M. Groesbeck.
1929. A study of methods for the isolation of *Bacterium abortus*. In: Undulant Fever Symposium. *p. 25–28.* Amer. Pub. Health Assoc. New York, N. Y.

[11] Hagan, William A.
1922. Studies on the disease of guinea pigs due to *Bacillus abortus*. Jour. Exp. Med. 36:697–709.

[12] Hagan, William A.
1922. The value of heat killed cultures for the prevention of the *Bacillus abortus* inoculation disease of guinea pigs. Jour. Exp. Med. 36:711–725.

[13] Huddleston, I. Forrest, D. E. Hasley, and J. P. Torrey.
1927. Further studies on the isolation and cultivation of *Bacterium abortus* (Bang). Jour. Infect. Diseases **40**:352–368.

[14] Jaffe, R. Hermann.
1922. Über die experimentelle Infektion des Meerschweinchens mit dem *Bac. melitensis* (Bruce) und dem *Bac. abortus* (Bang). Arch. Path. Anat. u. Physiol. (Virchow) **238**:119–134.

[15] Nelson, J. B.
1926. A rapid method for the isolation of *Bacillus abortus* from uterine exudate and diseased placenta. Jour. Exp. Med. **43**:331–338.

[16] Schroeder, E. C., and W. E. Cotton.
1911. The bacillus of infectious abortion found in milk. 28th Ann. Rpt. U. S. Dept. Agr. Bur. of Anim. Ind. *p. 139–146.*

[17] Seddon, H. R.
1915. Some observations on the methods of using the agglutination test in the diagnosis of the disease in bovines caused by the bacillus of contagious abortion. Jour. Compar. Path. and Ther. **28**:20–36.

[18] Smith, T., and M. Fabyan.
1912. Über die pathogene Wirkung des *Bacillus abortus* Bang. Centrbl. Bakt. (etc.) Abt. I, Orig. **61**:549–555.

[19] Soule, M. H.
1930. Bacteriological and serological findings in *Brucella abortus* infections in animals and man. Premier Congres International de Microb. **1**:606–607.

[20] Surface, Frank M.
1912. Bovine infectious abortion, epizootic among guinea pigs. Jour. Infect. Diseases **11**:464–467.

[21] Traum, J., and B. S. Henry.
1930. Boric acid for the preservation of milk naturally infected with *Brucella abortus.* Jour. Infect. Diseases **47**:380–383.